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Abnormal Expression of Four Novel Molecular Markers Represents a Highly Aggressive Phenotype in Breast Cancer. Immunohistochemical Assay of p53, nm23, erbB-2, and Cathepsin D Protein

SEHWAN HAN, MD,[1]* IK-JIN YUN, MD,[2] DONG-YOUNG NOH, MD,[2] KUK-JIN CHOE, MD,[2] SANG-YONG SONG, MD,[3] AND JE G. CHI, MD,[3]

[1]*Department of Surgery, Inje University Sanggye Paik Hospital, Seoul, South Korea*
[2]*Department of Surgery, Seoul National University College of Medicine, Seoul, South Korea*
[3]*Department of Pathology, Seoul National University College of Medicine, Seoul, South Korea*

Background: In view of the cumulative results to date, p53, nm23, erbB-2, and cathepsin D are the most promising investigational prognostic factors in breast cancer.

Objectives: The clinical utility of these molecular markers to predict recurrence was evaluated.

Methods: Archival pathology tissues of 100 breast cancer patients were analyzed by immunohistochemical assay. Molecular biologic data were merged with clinicopathologic variables.

Results: Thirty-two patients (32%) had recurrence of disease at a median follow-up of 48 months (range 26–72 months). Investigational factor expression had statistical correlation for recurrence with increasing coexpression: one variable 20.6%, two variables 34.2%, three variables 47.1%, four variables 80.0% ($P = 0.003$). In univariate analysis, lymph node metastasis, tumor size, erbB-2 protein overexpression, and loss of nm23 protein expression were significant variables to determine recurrence; in multivariate analysis, node status and tumor size emerged as the most significant variables for recurrence.

Conclusions: Coexpression of the studied investigational variables functioned as significant prognostic correlates for recurrence. These findings suggest that the studied investigational prognostic factors possess the ability to discriminate a highly aggressive phenotype in breast cancer.

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KEY WORDS: prognosis; recurrence; metastasis; survival

INTRODUCTION

Decision making for adjuvant chemotherapy depends on pathologic stage, estrogen (ER) or progesterone receptor (PgR) status, and the degree of differentiation of breast carcinoma [1,2]. A few studies reported that there was no significant survival benefit in patients with ER expression, thus ER status is regarded as the index of responsiveness to hormone therapy [3,4]. Although conflicting results have appeared in the literature, the large studies with longer follow-up consistently demonstrate that patients with ER/PgR-positive tumors have longer disease-free intervals than patients with ER/PgR-negative tumors [5,6]. Differentiation grade of breast carcinoma, histologic or nuclear, is an effective tool as a part

*Correspondence to: Sehwan Han, MD, Department of Surgery, Inje University Sanggye Paik Hospital, 761-1, Saggye-Dong, Nowon-Gu, Seoul, 139-707, South Korea. Fax: 82-2-938-4109; e-mail:shwhan@unitel.co.kr

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of routine histologic diagnostic work-up. However, it is not a reliable prognostic parameter because of the discrepancy among examiners [7,8]. Tumor size and axillary lymph node status at the time of dissection are still the most accurate prognostic parameters to predict the probability of recurrence in the patients with breast carcinoma.

Numerous investigational prognostic factors, mainly oncogene or tumor suppressor gene derivatives, are under clinical trial to complement and improve the aforementioned established clinical parameters. However, reproducibility of assay techniques has been quite poor, adding to the difficulty in universal application of newly developed molecular markers for prediction of outcome [9]. It is clear that a single alteration of one gene does not result in cancer. It seems likely that for each cancer, either a different combination of genetic alterations are involved or the same alterations lead to differing phenotypes depending on target tissue specificity.

p53 is a nuclear phosphoprotein normally expressed at very low levels in all human cells and regulates cell growth and division [10]. It is overexpressed in 25–50% of primary breast carcinomas and is the most commonly altered genetic change active in human cancer [11,12]. The nm23 gene and its product are known to be inversely related to the metastasizing potential of tumor cells [13]. Reduced expression of nm23 protein has been observed in experimental animal systems as well as in human breast cancers [14]. Currently, the nm23 gene is a representative tumor suppressor gene which exhibits downregulation of metastatic potential in cancer cells [15]. erbB-2 protein is structurally similar to epidermal growth factor receptor (EGFR) and is a member of the tyrosine kinase receptor family. It is located on chromosome 17q and is amplified or overexpressed in several human cancers [16]. About 20–25% of breast cancers have amplified erbB-2, but the rate increases to 40% when node-positive cancers are included [17–19]. In the patients with node-positive cancers, it has been reported that amplification of erbB-2 is associated with poor prognosis and frequent relapse [20]. Cathepsin D, estrogen-regulated protease, was originally identified in breast cancer cells as a 52 KD protein [21]. It is proposed that cathepsin D facilitates cancer cell migration and invasion by digesting the basement membrane, extracellular matrix, and connective tissues [22,23]. Cathepsin D status was a good predictor of metastatic disease in several clinical series [24–26].

Among the numerous molecular prognostic factors, p53 and nm23 are tumor suppressor genes, while erbB-2 is the most extensively studied oncogene in breast cancer. Cathepsin D and nm23 have been investigated for their role in breast cancer metastasis. Using immunohistochemical methods, we analyzed the relationship between the overexpression of p53, erbB-2, and cathepsin D protein together with decreased expression of nm23 protein and clinicopathologic parameters in patients with breast cancer who underwent surgery. We also correlated abnormal expression of these molecular markers with recurrence to assess their significance as prognostic variables in breast cancer.

TABLE I. Clinical Characteristics of Breast Cancer Patients Included in the Immunohistochemical Analysis of Molecular Variables

Characteristics	No. of patients (n = 100)
Tumor size (cm)	
⩽2	20
2–5	59
>5	21
Axillary lymph node status	
0	43
1–3	23
⩾4	34
Stage	
I	19
IIa	21
IIb	18
IIIa	24
IIIb	18
Estrogen receptor status	
Negative	41
Positive	59
Nuclear grade	
I	47
II	41
III	12
Age (years)	
⩽50	63
>50	37

MATERIALS AND METHODS

The medical records and archival pathology tissues from 100 breast cancer patients treated surgically at Seoul National University Hospital (SNUH) were evaluated. Patients with stage I (n = 19), stage IIa (n = 21), stage IIb (n = 18), stage IIIa (n = 24), and stage IIIb (n = 18) cancer between 1988 and 1993 were included in the study. Important selection criteria were abundance of tissue to allow immunohistochemical analysis and availability of follow-up study. Forty-three patients (43%) had negative nodes and 57 (57%) had positive nodes. ERs were expressed in 59 patients (Table I). All patients were treated with either modified radical mastectomy (n = 89) or lumpectomy with axillary lymph node dissection (n = 11). Postoperative adjuvant therapy, which was mainly applied to the patients with stage II and III breast cancer, included chemotherapy, radiation therapy, and tamoxifen. Thirty-two patients had recurrence of disease at completion of this analysis with a median follow-up period of 48 months (range 26–72 months).

The paraffin blocks for these 100 patients were re-

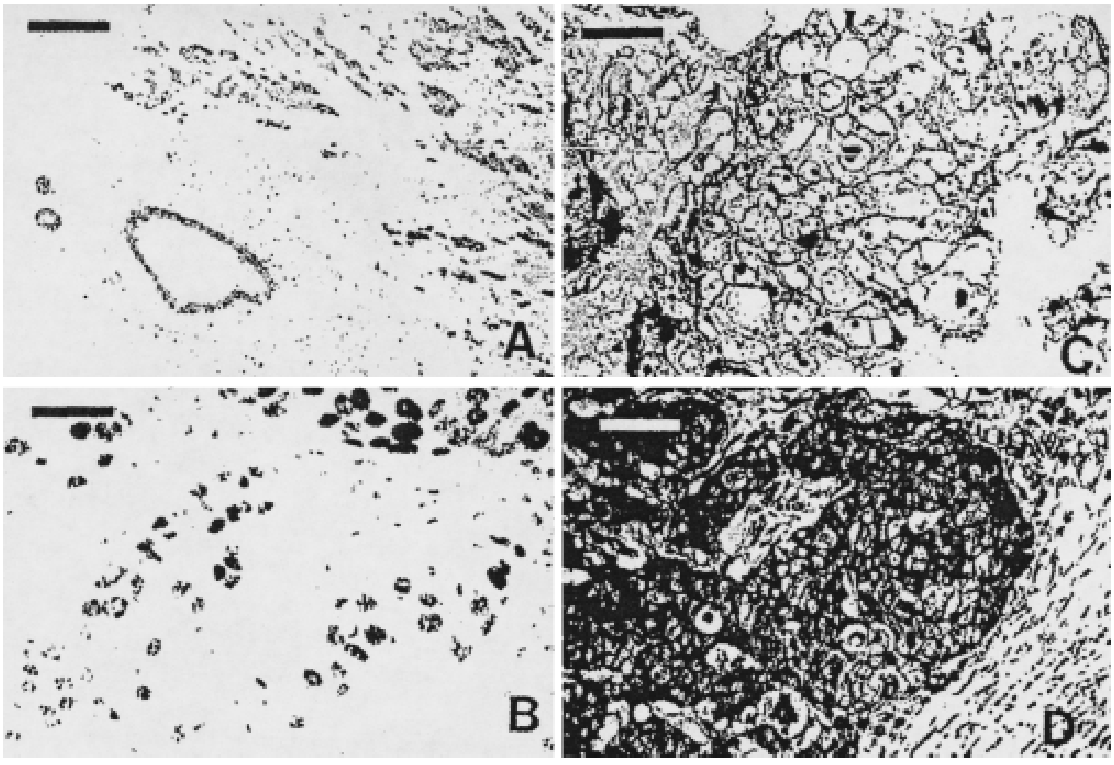

Fig. 1. Positive immune staining of molecular markers. **A:** Granular cytoplasmic staining of nm23 protein; bar scale is 100 μm. ×100. **B:** Dense granular staining of p53 protein in nuclei of cancer cells; bar scale is 25 μm. ×400. **C:** Granular staining of erbB-2 protein in cancer cell membranes; bar scale is 25 μm. ×400. **D:** Lysosomal staining of cathepsin D in cancer cells; bar scale is 25 μm. ×400.

trieved. Paraffin-embedded neoplastic tissues of these breast cancers were examined for expression or coexpression of p53, nm23, erbB-2 proteins, and cathepsin D by means of the avidin-biotin complex (ABC) immunoperoxidase method. We used commercially available antibody kits; DAKO-p53, DO-7 for p53 protein assay (DAKO Co., Glostrup, Denmark); NCL-nm23/NDPK-A protein for nm23 protein assay (Novocastra Laboratories Ltd., Newcastle-upon-Tyne, U.K.); monoclonal anti-erbB-2 for erbB-2 protein assay (Novocastra Laboratories Ltd.); and DAKO polyclonal rabbit anti-cathepsin D for cathepsin D assay (Dako Co.). Counterstaining with hematoxylin was done after ABC immune staining and two pathologists evaluated immunohistochemical staining without information of patients' outcome data. Two pathologists reviewed the slides together if interpretation of the immunohistochemical analysis differed. Three separate blocks containing malignant cells were stained and scored. Sections of breast carcinoma observed to express homogenous and/or intense immunohistochemical staining for the studied molecular marker proteins in more than 10% of the observed field were considered to be positive for overexpression (Fig. 1).

Molecular biological data were merged with clinicopathologic demographics to determine the frequency of single or combined expression in patients with recurrent disease, and to evaluate these molecular markers for their ability to predict recurrence.

The statistical analyses were performed using personal computer statistics software (PC-SAS, 6.04 version package program). Correlation between prognostic parameters and investigational factors was estimated by Chi-square test. The Cox proportional hazard model was used for univariate and multivariate analysis of prognostic variables.

RESULTS

In the current study, a strong expression of p53 was detected in 41/100 (41%) breast cancer patients. The tissues exhibited intense nuclear staining of malignant cells in a histologic section (Fig. 1B). A significant association was found between p53 overexpression and metastatic spread to lymph nodes (Table II). With poorly differentiated carcinomas, a greater proportion of tumors 8/12 (66.7%) were p53 positive, whereas 15/47 (31.9%) of the patients who had well-differentiated carcinomas expressed high levels of p53 protein (Table II).

nm23 expression decreased in 61% of 100 patients, and significantly correlated with tumor size and lymph node metastasis ($P = 0.036$). Only dense granular cytoplasmic staining of nm23 protein was interpreted as normal (Fig. 1A). Loss of nm23 expression was apparent

TABLE II. Correlation of Investigational Prognostic Factor Expression With Established Prognostic Factors in 100 Breast Cancer Patients

Variables	Total	p53	nm23	erbB-2	Cathepsin D
Tumor size (cm)					
⩽2	20	10 (50.0)	11 (55.0)*	4 (20.0)*	9 (45.0)
2–5	59	20 (33.4)	20 (33.4)	22 (37.3)	26 (44.1)
>5	21	11 (52.4)	8 (38.1)	10 (47.6)	7 (33.3)
Node status					
0	43	17 (39.5)	23 (53.5)*	17 (39.5)	19 (44.2)
1–3	23	6 (26.1)	8 (34.8)	10 (43.5)	8 (34.8)
⩾4	34	18 (52.9)*	8 (23.5)	9 (26.5)	15 (44.1)
Estrogen receptor status					
Negative	41	15 (36.6)	13 (31.7)	15 (36.6)	13 (31.7)
Positive	59	26 (44.1)	26 (44.1)	21 (35.6)	29 (49.2)
Nuclear grade					
I	47	15 (31.9)	25 (53.2)*	18 (38.3)	20 (42.6)
II	41	18 (43.9)	11 (26.8)	13 (31.7)	17 (41.5)
III	12	8 (66.7)*	3 (25.0)	5 (41.7)	5 (41.7)
Age (years)					
⩽50	63	30 (47.6)	26 (41.3)	21 (33.3)	24 (38.1)
>50	37	11 (29.7)	13 (35.1)	15 (40.5)	18 (48.6)

*$P < 0.05$ by Chi-square test.

with larger tumor size, and also increased as the number of involved lymph nodes increased (Table II). Decreased expression of nm23 protein was evident in the patients with poorly differentiated carcinoma ($P = 0.042$).

Overexpression of erbB-2 protein, which was densely stained in the cancer cell membrane (Fig. 1C), exhibited significant correlation with large tumor size only ($P = 0.039$), whereas cathepsin D had no significant correlation with other clinicopathologic parameters (Table II).

Thirty-two patients (32%) had recurrent disease during the follow-up period (median 48 months). When the frequency of overexpression or decreased expression of molecular protein markers was compared between the patients with recurrent and controlled disease, erbB-2 protein was overexpressed significantly in the patients with recurrent disease ($P = 0.042$). There was also an increasing tendency in the overexpression of p53 protein and cathepsin D with recurrent disease (Table III). Expression of nm23 protein decreased significantly in patients with recurrent disease ($P = 0.011$).

There was strong statistical correlation between the number of patients with recurrence and overexpression or loss of expression of molecular markers. When two molecular markers were expressed abnormally, recurrence was observed in 34.2%. However, the recurrence rate increased to 47.1% and 80.0% when three and four molecular markers, respectively, were expressed abnormally (Table IV).

In univariate analysis, lymph node metastasis, tumor size, erbB-2 protein overexpression, and decreased expression of nm23 protein were significant variables to determine recurrence; in multivariate analysis, node status and tumor size emerged as the most significant variables for prediction of recurrence (Table V). Overexpression or decreased expression of molecular markers exhibited borderline significance. We also carried out multivariate analysis to determine whether these molecular markers correlated with overall survival, which is the more meaningful endpoint. However, only seven patients have been confirmed to have died of breast cancer at the end of this study. Six of seven patients had lymph node metastasis, and axillary lymph node metastasis was the only prognostic indicator for overall survival ($P < 0.05$).

DISCUSSION

The ultimate purpose for investigating new prognostic factors for breast cancer is to improve the survival of the patients with this disease. We can expect to provide the patients with more accurate information about their disease and with proper treatment modalities by analyzing numerous prognostic factors. Integration of traditional pathologic markers with oncogene proteins or enzymes should enhance the ability to predict recurrence or survival in patients with breast carcinoma.

A significant association between decreased expression of nm23 protein in primary breast carcinoma and lymph node metastasis has been shown in the present study. This altered expression suggests that mutation of nm23 occurs during progression or metastasis of breast carcinoma, and that it is present early after tumor initiation. Decreased expression of nm23 has been observed in ductal carcinoma in situ (DCIS), especially with the comedo type [27]. In the current study, univariate analysis showed that abnormal expression of the nm23 protein in primary breast cancer was a significant variable to predict the risk for recurrence. It could be postulated that mutation of nm23 provides cancer cells with a significant

TABLE III. Comparison of Investigational Prognostic Factor Expression Between Disease-Free Group and Group With Recurrent Disease in Patients With Breast Cancer

	All (n = 100)	No recurrence (n = 68)	Recurrence (%) (n = 32)	*P* value
Cathepsin D	42	27 (39.7)	15 (46.9)	0.084
erbB-2	36	21 (30.9)	15 (46.9)	0.042
p53	41	26 (38.2)	15 (46.9)	0.078
nm23[a]	39	37 (54.4)	24 (75.0)	0.011

[a]Proportion of nm23 means loss of protein expression.

TABLE IV. Correlation of Investigational Prognostic Factor Expression to Recurrence in Breast Cancer

No. of (+) variables[a]	(+)/Total	Recurrence/(+) (%)
One variable (+)	34/100	7/34 (20.6)
Two variables (+)	38/100	13/38 (34.2)
Three variables (+)	17/100	8/17 (47.1)
Four variables (+)	5/100	4/5 (80.0)*

[a]Criteria for (+) variable is overexpression of p53, c-erbB-2, and cathepsin D protein whereas loss of expression is regarded as (+) variable in case of nm23 protein.
*$P = 0.003$.

TABLE V. Relationship of Prognostic Factors to Disease-Free Survival in Breast Cancer Patients

Variables	Univariate analysis	Multivariate analysis
Tumor size	0.0346	0.0421
Axillary lymph node metastasis	0.0112	0.0134
Estrogen receptor status	0.3841	0.1533
Nuclear grade	0.3685	0.4112
p53	0.4231	0.5345
nm23	0.0433	0.0512
erbB-2	0.0463	0.0532
Cathepsin D	0.0743	0.1010

growth advantage, allowing them to progress more quickly to an advanced stage.

The p53 gene is actually the single most commonly mutated gene in human cancer [28]. However, it is still controversial whether mutation of p53 provides us with prognostic information about the patients with breast carcinoma [29]. Genetic assay is the most accurate technique for identification of p53 mutations, but it is laborious and requires specially equipped molecular laboratories. From the clinical standpoint, p53 immunohistochemistry appears to be the most practical and useful method for detection of p53 alterations in carcinomas. This technique allows precise localization and identification of the cells that exhibit p53 alterations. However, a main obstacle to accepting immunohistochemistry results is that degradations during tissue transport and the handling procedure can alter the results. The difference in interpreting the results between laboratories is another problem to be solved [30]. Despite the partial association of p53 with markers of poor prognosis, p53 expression was not found to have prognostic value in this study. The results of the present study suggest that overexpression of the p53 protein cannot be an independent predictor of poor prognosis, although it reflects more aggressive phenotypes of breast carcinoma.

erbB-2 is the most widely investigated oncogene in breast carcinoma [31]. Its overexpression did not correlate with any traditional histopathologic parameters except tumor size in this study. In univariate analysis, patients with overexpression of the erbB-2 protein fared worse than did those with negative staining. According to several studies [17,20,32] erbB-2 is an accurate prognostic marker in patients with positive axillary lymph nodes. Gusterson et al. [33] described the prognostic importance of erbB-2 in 1,506 breast cancer patients from the International (Ludwig) Breast Cancer Study Group. They concluded that tumors with overexpression of the erbB-2 oncogene are less responsive to cyclophosphamide, methotrexate, and fluorouracil (CMF)-containing adjuvant therapy regimens than those with a normal amount of gene product. Of 43 node-negative patients, we had only 4 cases with recurrent disease, and CMF chemotherapy was the first-line regimen for the patients with axillary lymph node metastasis in the studied population. The results from the current study are consistent with these previous studies. The frequency of erbB-2 overexpression increased significantly in patients with recurrent disease in our study.

One of the remarkable findings in our study is an increased frequency of erbB-2 overexpression and loss of nm23 protein expression in the patients with recurrent disease. There was an increasing tendency of p53 and cathepsin D overexpression with recurrent disease. These results were associated with shorter disease-free survival in univariate analysis. We suggest from the results of this study that genetic alterations of oncogene or tumor suppressor genes are important in determining the biological aggressiveness of breast carcinoma. It is the current concept of carcinogenesis that neoplastic transformation consists of multiple accumulation of diverse genetic events [29].

Using immunohistochemical methods, we analyzed

the mutations of representative oncogenes, tumor suppressor genes, and proteases, the so-called investigational prognostic factors. In this study, abnormal expression of these investigational prognostic factors had statistical correlation for recurrence with increasing coexpression: one variable, 20.6%; two variables, 34.2%; three variables, 47.1%; four variables, 80.5% (Table IV). This result suggests that coexpression of molecular markers in neoplastic transformation endows cells of invasive breast carcinoma with an aggressive phenotype. Coexpression of studied molecular markers functioned as significant prognostic correlates for recurrence, even though genetic alteration of a single molecular marker did not possess independent predictive value for recurrence.

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